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Determination of nitrofuran metabolites in poultry muscle and eggs by liquid chromatography-tandem mass spectrometry

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Abstract

The use of nitrofurans in food-producing animals has been banned in EU. Detection of the protein-bound nitrofuran metabolites is the best approach to evaluate their utilization. A fast, sensitive and reliable LC–MS–MS method is presented to analyze simultaneously the metabolites of four commonly used nitrofuran drugs, furazolidone, furaltadone, nitrofurazone and nitrofurantoin. The sample clean up was performed by a single liquid–liquid extraction step, after a hydrolysis and derivatisation process. Separation of the molecules was performed by liquid chromatography in a C18 column (100 mm × 2.1 mm, 4 μm) at room temperature. The quantitative and confirmatory determination of these metabolites was performed by multiple reactions monitoring (MRM). Limits of quantification of 0.5 ng g^{-1} were achieved and the total analysis was accomplished in 5 min. This protocol has been applied to identify contaminated samples of poultry muscle and egg products.

Keywords: Nitrofuran metabolites; Poultry muscle; Eggs; Mass spectrometry

1. Introduction

Furazolidone, furaltadone, nitrofurazone and nitrofurantion are veterinary drugs that belong to the nitrofuran group, which have been used in the treatment of infections caused by *Escherichia coli* and *Salmonella* in pigs, poultry and fishes. The nitrofurans are quickly metabolized and are not detected after few hours from their administration. Otherwise, nitrofuran metabolites remain during months as residues bound to tissue proteins.

It has been demonstrated that a proportion of the bound residues of furazolidone [1] and furaltadone [2] possess intact side-chains which have molecular characteristics in common with the parent compounds. These side-chains can be released from the bound metabolites under mildly acidic conditions such as may occur in the stomach of the consumer. It has been suggested that furazolidone side-chain, 3-amino-2-oxazolidinone (AOZ), can be metabolized into β-hydroxyethylhydrazine, which is a mutagenic and carcinogenic compound [2]. Because no safe limit for the presence of these drugs in food product for human consumption could be assigned, nitrofurans have been banned for food-producing animals by the European Union (EU) in 1995 [3].

The bound metabolites are highly stable (between 4 and 9 days half-life time) and their detection is still possible when concentrations of the parent drugs are below the detection limits. These metabolite analysis have been developed upon the determination of their 2-nitrobenzaldehyde imine-type derivatives with UV and mass spectrometric detection [4,5–9]. Otherwise, only AOZ [4,6,7] and 3-amino-5-morfolinomethyl-2-oxazolidinone (AMOZ) [9] have been analysed by mass spectrometry until recently. Leitner et al. reported a LC–MS–MS method that detects simultaneously the metabolites of the four nitrofurans already mentioned in different animal tissues [10]. Sample clean-up and analyte enrichment was performed by solid-phase extraction (SPE) and limits of detection of 0.5–5 ng g^{-1} were achieved using eletrospray ionization in positive mode [10].

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It was demonstrated that the concentrations of furazolidone and AOZ reached around 360–380 ng g^{-1} and AOZ is a more suitable marker residue than the parent drug for monitoring nitrofurans in eggs, because of its stability [11]. As a high level of AOZ nitrofuran metabolite has been found in eggs, the investigation of other nitrofuran residues presence became important.

The Brazilian Agricultural Ministry created a nitrofuran analysis program to detect the presence of residues in animal food-products to avoid exportating of contaminated samples. Therefore, it was our major goal to improve the methods of nitrofuran metabolites detection, already described [4–10], providing an easier and faster analysis of a large number of samples, with high confidence at a very low concentration range. After hydrolysis and derivatisation process [10] a one-step liquid–liquid extraction was applied, which decreases the expended time related to the SPE. Two deuterated internal standards (AOZ-d4 and AMOZ-d5), were used to mimic the analytes extraction. A high sensitivity was achieved with tandem mass spectrometry in the API4000 equipment (PE Sciex, Canada), providing confident analyte identification in low concentrations.

2. Experimental

2.1. Chemicals and solvents

The metabolites AOZ, AMOZ and 1-aminohydantoin (AHD), the internal standards AOZ-d4 AMOZ-d5 and the analytes NPAOZ, NPAHD and NPSEM (Fig. 1) were synthesized by Seagoe Industrial State (Chemical Synthesis Services, Craigavon, Northern Ireland), purity of 95% or higher. SEM (semicarbazide) was supplied by Sigma (Aldrich Chemical Company, Germany). Stock solutions were prepared in methanol and stored at 4 °C for a maximum of 1 month.

Methanol (HPLC grade) and ethyl acetate (HPLC grade) were obtained from Mallinckrodt (Mallinckrodt Chemicals, USA), hydrocloric acid by Mallinckrodt (Mallinckrodt Baker, SA, Mexico). Tri-sodium phosphate dodecahydrate (p.a.) and sodium hydroxide (p.a.) were obtained from Synth (Labsynth Produtos para Laboratório Ltd., Brazil). Water was purified, using the Milli-Q or Elga UHQ systems, prior to use. 2-Nitrobenzaldehyde was supplied by Sigma (Aldrich Chemical Company, Germany).

2.2. LC–MS–MS analysis

The LC–MS–MS system consisted of LCADVp Liquid Chromatograph Shimadzu System (Shimadzu Coorporation, Japan) connected to a PE Sciex API 4000 triple quadrupole mass spectrometer (PE Sciex, Toronto, Canada) in eletrospray positive ionisation mode. A CTC HTS PAL autoinjector was connected to the system.

The chromatography was performed in a C18 (100 mm × 2.1 mm, 4μm) Jones®, connected to a C18 (1 cm × 4 mm, 4 μm) Jones® pre-column. The mobile phase was composed by two solutions: A (water and 0.1% acetic acid) and B (90% acetonitrile, 10% water and 0.1% acetic acid) in a gradient that started with 60% of A and 40% of B; during the first min the concentration of B was decreased to 10%; from 1 to 3 min the concentration of B was raised to 90%. Finally, from 4 to 4.5 min the B concentration was decreased to 10%. The column was operated at room temperature at a flow rate of 0.45 ml min^{-1}.

The ions were monitored by Multiple Reaction Monitoring (MRM) according to described by Leitner et al. [10], exception of NPAOZ, that the product ions were 134 and 104 (*m*/*z*). The source block temperature was set to 450 °C and the eletrospray capillary voltage to 4.5 kV.

2.3. Sample preparation

A 1 ± 0.05 g portion of each sample was transferred to a 15 mL centrifuge tube. The samples were submitted to hydrolysis and derivatisation processes, by adding 40 μL of internal standard mixture (50 ng/mL of AM0Z-d5 and l00 ng/mL of AOZ-d4), 5 mL of 0.2 M hydrochloric acid and 50 μL of l00 mM 2-nitrobenzaldehyde (2-NBA) [10], under UV light protection. The samples were placed in a shaker at 130 rpm and incubated overnight at 37 ± 2 °C.

After incubation time the samples were removed from the shaker and allowed to lower and stabilize the temperature. To adjust the samples pH to 7 ± 0.5, 500 μL of 0.3 M trisodium phosphate dodecahydrate was added followed by about 400 μL of 2 M NaOH. Derivatised residues were extracted by adding 4 mL of ethyl acetate and mixing them in an orbital shaker for 30 min at 130 rpm at room temperature. The samples were centrifuged for 10 min at 3250 × *g* and the organic layer was transferred to a glass tube and let evaporate to dryness at 45 °C under a mild flow of nitrogen in an evaporation station. The residues were redissolved in 500 μL acetonitrile–water (10:90, v/v) and 0.1% acetic acid mixture (reconstitution solvent) and centrifuged at 12,200 × *g* for 5 min. For poultry muscle samples, the non-turbid layer was collected and transferred to a HPLC vail. For egg samples, the upper layer (a fat layer) was removed and the samples were centrifuged again at 12,200 × *g* for 5 min, before transferring to a HPLC vail.

2.4. Calibration curve and determination of limit of quantification (LOQ)

A calibration curve was prepared with blank samples which were fortified with a standard solution mixture (AOZ, AMOZ, AHD and SEM at 50 ng/mL), to analyte final concentrations of 0.3, 0.5,1.0, 2.0 and 5.0 ng g^{-1}, prepared in duplicate.

Twenty blank samples of either poultry muscle or eggs were fortified with the standard solution mixture to a final

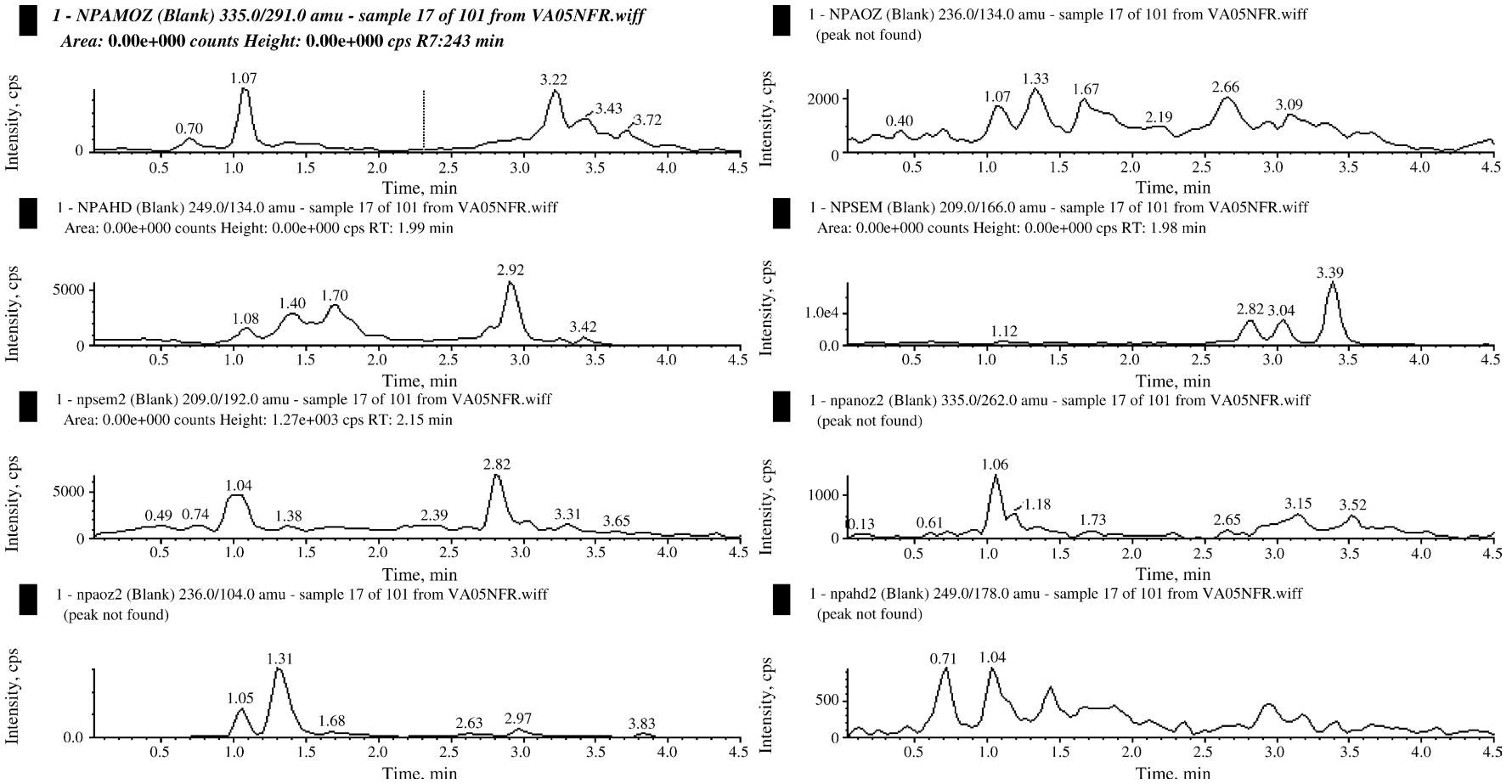

Fig. 1. MRM chromatograms of blank and spiked poultry muscle samples. The spiked samples contained 0.5 ng g^{-1} of each analyte.

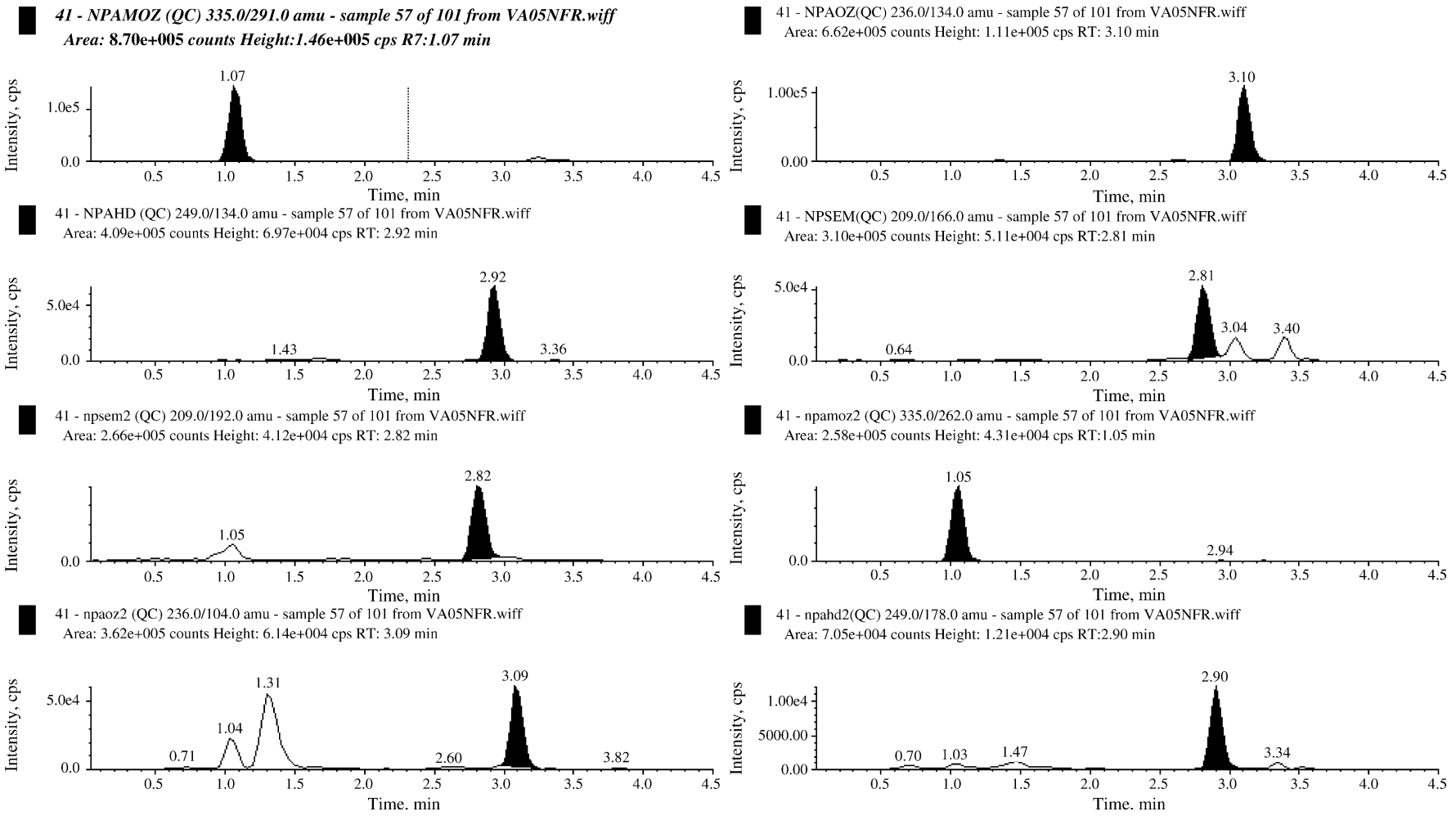

Fig. 1. (*Continued*).

concentration of 0.5 ng g^{-1}. The internal standard AMOZ-d5 was used for AMOZ quantitation and AOZ-d4 was used for the others.

2.5. Recovery experiments

Six blank samples were fortified with a standard solution mixture to a final concentration of 0.50, 0.75 and 1.0 ng g^{-1} (total of 18 samples). These samples were extracted according to the method described in item 2.3. Other six blank samples were fortified with a derivatised standard analyte solution mixture (NPAOZ, NPSEM and NPAHD at 50 ng/mL) after extraction, to a final concentration of 0.50, 0.75 and 1.0 ng g^{-1} (total of 18 samples). This mixture was used to include the derivatisation efficiency in the recovery determination. A calibration curve was set with reconstitution solvent aliquots fortified with analyte solution mixture, to evaluate the matrix effect. Other experiments were similarly set using poultry muscle to evaluate the recovery of the analytes in different conditions: extraction under UV light protection and extraction under UV light protection after two extraction cycles with ethyl acetate.

3. Results and discussion

3.1. Optimization of the method

The liquid–liquid extraction is known to be less clean as SPE protocol, but has the advantage of being cheaper and easier to handle. Besides this, the time consuming was decreased comparing to other liquid–liquid extraction protocols, since we replaced the traditional two extraction cycles with ethyl acetate [6] for just one extraction cycle without compromising the recovery. Using three different liquid–liquid extraction conditions, we obtained similar recoveries for all the analytes in poultry samples. However, different analytes were not recovered at the same extent when compared to each other inside the same protocol. We determined a 30% recovery for NPAOZ and NPSEM while a very high recovery was observed for NPAHD. Even at lower recovering level as in the case of NPAOZ and NPSEM, our method showed a very good reproducibility presenting variability among samples below 15% (Table 1). This recovery level was observed due the high matrix effect, around 30% at LOQ level for NPSEM and 60% for NPAOZ.

The proposed extraction protocol was followed to determine nitrofuran residues in eggs. A lower NPAHD recovery percentage was obtained for this kind of matrix when compared to poultry matrix (Table 1). Otherwise, the variability among the extracted samples for the analytes recovery was below 15%, which shows again a good reproducibility of the method.

The chromatographic system applied in our method exhibited enough resolution to separate the analyte peaks from those resulting from matrix interference, even with an analytical run of 5 min, while in the methods already described, this time was at least 10 min [10]. Some matrix interference could be detected near the NPSEM retention time, which was excluded by using two ion products monitoring (*m*/*z* of 166 and 192).

Some authors reported that the sensitivity for NPAHD and NPSEM was always lower that observed for NPAOZ and NPAMOZ [6,9,10]. Our method enabled analysis with very high sensitivity for all four analytes, even in the range around our limit of quantification (0.5 ng g^{-1}). This sensitivity was achieved for poultry muscle (Fig. 1) and egg as well.

3.2. Method validation and discussion

To validate data among experiments (inter-batch validation) six blank samples were fortified with a standard analyte solution to a final concentration of 0.50, 0.75 and l.0 ng/mL (total of 18 samples). The results were compared to other two validation batches, which were prepared by different analysts, in different days and with different solutions. This protocol

Table 1
Recovery for NPAHD, NPAOZ and NPSEM in poultry muscle, dried egg and in natura egg samples

Nominal concentration (ng/mL)	Recovery for NPSEM (%)	NPSEM CV[a] (%)	Recovery for NPAHD (%)	NPAHD CV[a] (%)	Recovery for NPAOZ (%)	NPAOZ CV[a] (%)
						Poultry
0.5	43.2	8.7	129.4	3.7	47.6	14.0
0.75	28.1	6.0	104.2	5.8	30.3	14.6
1.0	28.3	3.9	104.3	10.5	34.3	8.5
						Driedegg
0.5	35.0	2.3	68.3	6.9	34.7	5.2
0.75	33.8	3.0	61.1	3.4	30.8	10.2
1.0	31.5	6.0	63.7	6.9	30.7	5.3
						Innaturaegg
0.5	19.6	4.5	31.5	4.4	14.5	4.4
0.75	20.7	1.4	33.6	4.5	19.4	4.9
1.0	19.5	2.6	33.9	6.3	18.4	6.2

[a] CV, coefficient of variability among the six extracted samples (standard deviation/mean) × l00.

Table 2
Inter batch validation data for poultry matrix

	Mean	Standard deviation	Accuracy (%)	CV (%)
0.5 ng g^{-1} (nominal concentration)				
NPAHD	0.54	0.05	107.2	9.6
NPAOZ	0.54	0.06	107.0	10.6
NPAMOZ	0.56	0.06	111.6	10.1
NPSEM	0.53	0.05	105.2	9.1
0.75 ng g^{-1} (nominal concentration)				
NPAHD	0.79	0.07	105.2	9.3
NPAOZ	0.79	0.05	105.6	6.4
NPAMOZ	0.80	0.06	106.9	7.6
NPSEM	0.78	0.06	103.5	8.0
1.0 ng g^{-1} (nominal concentration)				
NPAHD	1.09	0.06	109.3	5.9
NPAOZ	1.06	0.07	106.1	6.7
NPAMOZ	1.08	0.07	107.6	6.6
NPSEM	1.02	0.05	102.0	5.0

Mean, standard deviation, accuracy and CV were calculated from data of three independent validation batches. Each validation batch was composed of 18 blank samples fortified at analyte final concentrations of 0.5, 0.75 and 1.0 ng g^{-1}.

Table 3
LOQ validation data for poultry matrix

0.5 ng g^{-1} (nominal concentration)	Mean	Standard deviation	Accuracy (%)	CV (%)
NPAHD (249/134)	0.48	0.04	95.6	8.9
NPAOZ (236/134)	0.48	0.03	96.3	6.0
NPAMOZ (335/291)	0.49	0.02	97.3	4.2
NPSEM (209/166)	0.46	0.04	91.6	8.7
NPAHD (249/178)	0.49	0.04	98.7	7.6
NPAOZ (236/104)	0.48	0.02	95.9	4.6
NPAMOZ (335/262)	0.49	0.02	97.5	3.6
NPSEM (209/192)	0.45	0.04	90.3	8.3

Twenty blank samples were fortified with AOZ, AMOZ, AHD and SEM to a final concentration of 0.5 ng g^{-1}. These samples were analyzed according to the proposed method.

was carried out using poultry muscle, dried egg and in natura egg. An accuracy between 70 and 130% and a coefficient of variability (CV) lower than 20% were found for all the experiments with the poultry matrix (Table 2), which indicates that the method is repeatable and reproducible, according to internationally accepted guidelines [12].

Methods already described in literature have reached detection limits of 0.5 ng g^{-1} in poultry samples [10]. The limits of quantification already reported have been in the range of 2.5 ng g^{-1} for AOZ and AMOZ and l0 ng g^{-1} for AHD and SEM [10]. In eggs, only AOZ has already been evaluated and its detection limit was 1 ng g^{-1} [11]. The limit of quantification (LOQ) of the present method was equal to other methods limit of detection, 0.5 ng g^{-1}, for poultry (Table 3) and egg samples (data not shown). The limit of detection observed for AMOZ was around 0.1 and 0.2 ng g^{-1} for all the other analytes.

4. Conclusion

An easy sample preparation protocol has been performed, including a one step liquid-liquid extraction that showed to be clean enough to attend our major goal, which was to create a simple method that could accommodate a large number of analyses in a short period of time.

The LC–MS–MS method here reported allows the simultaneous analysis of the all four nitrofuran metabolites (AOZ, AMOZ, AHD and SEM) in an analytical run of 5 min, with a high level of reliability. This method could be applied to analyze these residues in other matrix such as eggs.

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